Bacterial vectors for active immunotherapy reach clinical and industrial stages

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Active immunotherapy based on live attenuated bacterial vectors has matured in terms of industrial development and develops through a combination of three phenomena. First, active immunotherapy that stimulates an antigen-specific cytotoxic T-cell immune response has become a reality after several years of work. Second, there is still a need to identify vectors that can deliver antigens to the cytosol of antigen-presenting cells in vivo. Third, the recent progress in the understanding of bacterial lifestyle and in developing genetic engineering tools has enabled the design of bioengineered bugs that are capable of delivering antigens. Here, we review the mechanisms by which clinical bacterial vectors deliver antigens into the cytosol of antigen-presenting cells and summarize the development strategy of the three identified firms in this field.

Why Have Bacterial Vectors Reached the Clinical and Industrial Stage?

Active cellular immunotherapy is emerging. Advances in basic immunology have led to an improved understanding of the interactions between the immune system, tumors and pathogens. To re-engage the immune system in its fight against "bad" cells, active immunotherapy focuses on the development of agents that activate the immune system to target and kill these cells. Strategies to stimulate effector immune cells include ex vivo or in vivo vaccination with specific antigens, treatment with cytokines (e.g., IL-2), enhancement of antigen presentation (by stimulation of toll-like receptors (TLRs), administration of dendritic cells (DCs) or the use of CD40-targeted agonistic antibodies) and blocking inhibitory signals (antibodies against CTLA4). At the moment, more than 200 active therapeutic vaccine trials across a wide spectrum of cancers and infectious diseases are listed at ClinicalTrials.gov. The first active immunotherapeutic approach that reached the market, in 2010, was Sipuleucel-T (Provenge, Dendreon), which consists of autologous peripheral blood mononuclear cells loaded ex vivo with a fusion protein of prostatic acid phosphatase (PAP) and granulocyte-macrophage colony-stimulating factor (GM-CSF) to treat patients with advanced prostate cancer.

Need for in vivo antigen delivery. Among the different strategies to stimulate the antigen-specific T-cell response, the ex vivo loading of DCs (as in Provenge's strategy) appears to be too difficult to generalize because of logistical problems. Rather, many firms have conducted clinical trials using in vivo strategies based on the development of vectors that deliver antigens or nucleic acids encoding antigens to the cytosol of antigen-presenting cells (APCs). These vectors include viruses, virus-like particles, plasmid DNA, peptides, chimeric proteins and bacteria.[1]

Bacterial lifestyles have become better understood, and molecular engineering has assisted in the development of bacterial vaccines. "Killed" or attenuated bacteria have long been used for prophylactic vaccines. Hence, extensive clinical data are available, and firms have expertise in preparing and formulating this kind of vaccine. Microbial pathogens were the

earliest non-surgical cancer treatment. In the nineteenth century, William Coley developed the first bacterial-based cancer treatment, which was composed of killed gram-positive streptococci and gram-negative *Serratia marcescens* injected directly into the tumor.[2] However, safety concerns and the advent of radiotherapy and chemotherapy treatments led to a decline in the use of this treatment. Nevertheless, live Bacillus Calmette-Guerin (BCG) is still in clinical use for bladder cancer treatment. In this kind of bacteria-mediated cancer immunotherapy, many of the antitumor effects induced by bacteria are nonspecific, including angiostatic effects induced by pro-inflammatory cytokines, such as IL-12 and IFN-gamma, direct tumor necrosis by tumor necrosis factor (TNF) α, induction of epitope spreading induced by the death of tumor cells from these indirect mechanisms and the direct killing of tumor cells infected by bacteria. An important molecular component of DCs that can be targeted for immunotherapy is the TLR, which recognizes microbes. Indeed, the activation of DCs is likely to contribute to therapeutic effects that are associated with the nonspecific activation of anti-tumor immunity by pathogen-associated molecular patterns (PAMPs), and several TLR ligands are currently being tested in clinical trials.[3]

Bacteria can also mediate antigen-specific responses if they are engineered to express and deliver antigens to the correct compartment, i.e. the cytosol of APCs, during the vaccination process which became, in this case, a controlled infection. The understanding of the molecular mechanisms whereby DCs augment the stimulation of naive T-cells is rapidly growing. Nevertheless, host perception of the level of danger linked to this infection directly influences the type and intensity of the immune response.[4] Because the challenge of CD8+ T-cell-mediated immunotherapy is to overcome tolerogenic events (the majority of tumor antigens bear strong similarity to self-proteins), bacterial vectors have the advantage of delivering both an antigenic message and a strong danger signal mediated by their PAMPs. PAMPs, such as lipopolysaccharide, lipoproteins, flagellin and DNA containing unmethylated

cytosine-phosphate-guanine (CpG), can bind to their respective TLRs on APCs, such as DCs, leading to upregulation of co-stimulatory molecules, the maturation and migration of APCs to secondary lymph nodes and the production of pro-inflammatory cytokines.

Over the past 40 years, advances in bacterial engineering, molecular biology and our understanding of pathogenic bacterial lifestyles have significantly accelerated the rational design of bacterial vectors that induce the in vivo delivery of heterologous antigens inside APCs. Bacterial vaccine vectors offer multiple advantages: (1) there are several well-characterized virulence-attenuating mutations; (2) the number, the amount and the in vivo location of antigen expression can be regulated; (3) multiple vaccine delivery routes are possible; and (4) they potently stimulate the innate and adaptive immune systems.

How to Deliver an Antigen Inside APCs by Means of a Bacterium in Vivo?

Currently, two major engineered but natural processes are being explored at preclinical or clinical stages to achieve the delivery of antigenic proteins inside APCs. The first is based on the use of the type III secretion system to deliver bacterial toxins to a cell's cytoplasm. The second uses pathogens with natural intracellular tropism. At the industrial development stage, the first strategy uses primarily Salmonella sp or *P. aeruginosa*, and the second uses *Listeria monocytogenes*.

Type III-mediated delivery. The type III secretion system (T3SS) is a critical virulence factor used by a broad range of pathogens (e.g., Salmonella, Shigella, Yersinia and Pseudomonas) to deliver toxins into the cytoplasm of host cells. Toxins are addressed to the T3SS thanks to a sort signaling sequence and when fusing an antigen of interest to this short sequence, recombinant protein can be engaged by the T3SS and delivered to the cytosol of DCs, which are often targeted by pathogens to disrupt the immune response and favor natural infection. Therefore, this strategy is an effective way to deliver antigens directly inside APCs.[5-9]

Intracellular microbes bearing antigens. *L. monocytogenes* is a facultative, intracellular, gram-positive rod that invades the intestinal mucosa and is captured by phagocytes. Listeria can modify phagosomes into large compartments termed spacious Listeria-associated phagosomes (SLAPS). Listeria secretes listeriolysin O (LLO) and phospholipase C, which induces the degradation of the phagolysosome membrane and the release of 5–10% of the bacteria into the cytoplasm, where they multiply and become motile via the expression of ActA, an actin polymerase that enables actin mobilization. The use of live recombinant strains of *L. monocytogenes* to deliver antigens began with the pioneering work of Dr Yvonne Paterson in Pennsylvania. Her work demonstrated that a recombinant *L. monocytogenes* that expressed and secreted the model antigen influenza virus nucleoprotein (NP) fused to LLO could efficiently infect APCs, and this expressed NP protein was processed by the endogenous antigen-processing pathway, facilitating the presentation of NP epitopes in the context of MHC class I molecules.[10] Since then, the *L. monocytogenes* platform has rapidly evolved to become one of the most efficacious approaches for antigen delivery for infectious diseases[11] and cancer (For a review see.[12])

Salmonella is a gram-negative bacterium and intracellular pathogen that causes salmonellosis. Its intracellular survival and replication are restricted to the endosomal compartment of eukaryotic cells, such as macrophages. Several publications have illustrated the use of live attenuated Salmonella strains to elicit mucosal and systemic immune responses against antigens from other infectious bacteria, viruses, tumor antigens or tumor-promoting growth factors.[13] Despite the numerous preclinical studies using salmonella strains, few have advanced to human clinical trials, and no clinical efficacy has been reported.

How to Transform a Pathogen into an Innovative Therapeutic Product?

Strategies for attenuating bacterial virulence. According to the

"patho-biotechnology" approach,[14] live strains of bacteria have to be engineered to significantly improve their clinical effectiveness (safety and efficacy) and technological robustness. The primary goal is to develop bacterial strains that retain their capacity to synthesize/deliver antigens and induce robust immune responses against antigens but exhibit reduced pathogenicity.

In this context, two main strategies have been explored: live attenuated bacteria and killed but metabolically active (KBMA) bacteria.

Live attenuated vaccines. Over the past few decades, advances in bacterial genetics combined with the emergence of improved molecular biology tools have allowed the dissection of the mechanisms of bacterial virulence and have specified the complexity of host-pathogen interactions.[15]

The use of well-defined, non-reverting attenuating deletions allows for the development of live attenuated bacteria with no risk of reversion to a virulent state. To date, few strains of attenuated live bacteria vaccines have proven acceptable for human use in preventing infectious diseases. Attenuation is primarily based on the deletion of genes involved in particular metabolic pathways or critical virulence factors. One of the best-characterized attenuations in a metabolic pathway is the deletion of the *aroA* gene, which encodes 3-phosphoshikimate 1-carboxyvinyltransferase, an enzyme that is involved in aromatic amino acid synthesis. This attenuation renders Salmonella, Listeria and Pseudomonas species auxotrophic for aromatic amino acids, lowering their capacity for in vivo replication and eliminating their pathogenicity.[6,16]

Multiple virulence-attenuated vaccine vectors have also been developed. For example, in Salmonella, the most widely studied strains have mutations in the *phoP/phoQ* regulator system or in Salmonella pathogenicity island 2 (SPI2). Salmonella virulence factors encoded in the *phoP* locus induce the formation of spacious vacuoles from phagosomes, which allows bacterial persistence and growth in these organelles. The most commonly used deletions to decrease the virulence of Listeria vectors are the *actA* deletion, which blocks its ability to spread from cell to cell, thus limiting bacterial dissemination, and the *inlB* deletion, which blocks the direct uptake of Listeria by hepatocytes, thus minimizing its potential to cause liver-related toxicity.[17]

It is important to maintain a subtle balance between minimal pathogenicity and maximal immunogenicity with live bacterial vectors because attenuation procedures could impact the course of infection and hence the immune response. For example, oral administration of a PhoP-null Salmonella mutant induces the innate immune response, whereas an *aroA* mutant elicits a stronger TH1-like T-cell response.[18] However, an engineered strain of *L. monocytogenes* that enhances the NLRC4 inflammasome is too attenuated and fails to induce protective immunity based on antigen-specific CD8+ T-cells.[19] To counteract the poor infectivity linked to attenuation, Wang et al. have demonstrated that satisfactory T-cell immune responses with an *aroA*-attenuated *P. aeruginosa* vector could be restored by mimicking the intrabody dissemination using multiple loci injections.[20]

Killed but metabolically active vaccine. The killed but metabolically active (KBMA) approach recently reviewed,[21] is based on the Intercept Blood system® developed by Cerus Corporation (Concord, CA) to inactivate undetected microbes contaminating plasma and platelet blood products.[22] Briefly, it consists of a combination of a synthetic psoralen (amotosalen hydrochloride, S59) and illumination with long-wave UV (UVA) light, which induces covalent monoadducts and crosslinks of DNA and RNA. Brockstedt et al. used this technology and envisioned "a new vaccine paradigm" in which they combined the safety of a killed vaccine with the potency of a live vaccine. To obtain such a result, they removed genes required for nucleotide excision repair (*uvrAB*), which is the primary mechanism by which bacteria repair psoralen-induced crosslinks. Accordingly, these gene deletions render microbial-based vaccines more sensitive to photochemical inactivation with amotosalen and UVA illumination. Therefore, the number of crosslinks required to "kill" the bacterium is very low (theoretically, only one crosslink is sufficient to block bacterial replication). However, bacterial genes can still be expressed, a fact that allows KBMA bacteria to retain the essential properties of live bacteria without the ability to multiply. Thus, this innovative vaccine approach, using a Listeria vectorial strain, has been demonstrated to elicit functional T-cells and long-term protective immunity that correlate with vaccine efficacy in mouse models of infectious diseases and cancer.[23]

Stable and strong heterologous protein expression. The use of bacterial systems for the production of heterologous recombinant proteins is widespread in academic and industrial sectors. Experience in the fields of molecular biology and biochemistry in codon optimization, cloning of genes, tag fusions to enable detection, secretion, co-expression of multiple proteins and genome modeling has contributed to the continuous improvement of bacterial vaccines. Plasmids are commonly used to express large quantities of recombinant proteins in bacterial hosts. However, plasmid maintenance in bacteria often requires antibiotic resistance markers, which have been discouraged in clinical applications by the Food and Drug Administration. Therefore, two strategies have been explored to produce antigen delivery systems without antibiotic resistance genes. The first approach involves plasmid stabilization using complementation systems or balanced lethal systems with the strain. The second strategy consists of inserting the heterologous gene into the bacterial chromosome either by homologous recombination[24] or by phage-specific insertion.[25]

The plasmid-based strategy has the advantage of achieving high copy numbers of heterologous genes. Mechanisms of complementation have been described previously. In *L. monocytogenes*, one mechanism is based on the complementation of the *pfrA*-deficient strain with an episomal copy of *pfrA*,[24] and the second method uses d-alanine racemase (*dal*) complementation.[26] In Salmonella, a variety of vectors have been developed based on complementation of genetic defects in the biosynthetic pathways for DNA precursors, amino acids and cell walls biosynthetic pathways (for a review see ref. 27). In plasmid-based balanced lethal systems, plasmids encode an essential protein that is required for bacterial growth and

replication, thereby forcing the bacterium to retain the plasmid.[28]

Chromosomal integration is an obvious solution to achieve the stable expression of heterologous genes, but the fact that this might entail a single copy of the transgene of interest could constitute a major drawback of this approach. The addition of multiple copies of genes of interest could potentially overcome this drawback.

Complex antigens can be delivered. We demonstrated that large proteins can be correctly secreted and translocated by the T3SS of *P. aeruginosa* when fused to the N-terminal domain of the natural exotoxin, ExoS.[29] In this system, expression was controlled by bypassing the natural regulation of T3SS expression using a strong IPTG-inducible promoter to govern the expression of the ExsA natural T3SS transcription activator protein in trans. Furthermore, using that platform, it will likely be possible to develop more sophisticated expression systems that would allow, for example, the expression and delivery of multiple antigenic proteins.[20] These properties suggest that immunization protocols with whole antigenic proteins are feasible and simultaneously provide a panel of putative mono- or multi-antigenic epitopes. This approach offers the potential for a broad spectrum of applications for vaccination, bypassing the limitations associated with the use of short peptides that are restricted to particular major histocompatibility complex (MHC) class I alleles.

Good manufacturing practices. One of the advantages of bacterial vaccine vectors is that bacteria can be easily grown and can be purified and processed inexpensively at an industrial level. However, gene deletion for attenuation often renders the bacteria more difficult to culture. Moreover, to satisfy current good manufacturing practices for drug production, bacteria need to be grown in a chemically defined medium. These two problems could be simultaneously solved if bacterial strains can be progressively adapted to grow in an optimized CD medium, as was achieved for the *P. aeruginosa* platform, CLIN-1.[20]

Stabilization/formulation. Each therapeutic agent must be stable during the period of time between its production and its administration to a patient. Moreover, it is important that reconstituting the product is easy at the time of delivery to the patient. With bacteria, it should be possible to achieve stabilization using special media or lyophilization. Here again, recent knowledge concerning the lifestyle of bacteria could aid the pharmaceutic development of live bacterial vectors. For example, in trans expression of the stress tolerance gene, *betL,* from *L. monocytogenes* increases resistance to relevant stressors, including osmo-, cryo- and baro-stress, and this gene has been shown to improve tolerance to spray- and freeze-drying in other bacterial strains.[30]

Which Companies Develop Bacteria-Based Active Vaccines?

Three companies developing live bacteria-based vehicles for in vivo antigen delivery inside APCs have been identified. Each of these companies uses engineered *L. monocytogenes* or *P. aeruginosa* strains. One project using *Salmonella typhimurium* was also started in 2008 (VION Pharmaceuticals) but appears to have been terminated.

ADVAXIS SA and ADUROBIOTECH SA have completed the most advanced trials with *L. monocytogenes.*

ADAVAXIS has 2 ongoing phase 2 trials with their ADXS-HPV product, which is a live Listeria vaccine that secretes the HPV-16 E7 antigen fused to a non-hemolytic fragment of the Lm protein listeriolysin O (LLO) (http://clinicaltrials.gov/ct2/show/NCT01116245?term=advaxis&rank=1 and http://clinicaltrials.gov/ct2/show/NCT01266460?term=listeria&rank=6). The results of the phase 1 trial indicate that the vaccine is safe and that an efficacy signal can be observed.[31]

ADUROBIOTECH is also conducting a phase 2 clinical trial with CRS-207, a live attenuated Listeria vaccine against mesothelin tumor-associated antigen, for use in pancreatic cancer (www.clinicaltrials.gov/ct2/show/NCT01417000?term=NCT01417000&rank=1). CRS-207 was evaluated in a phase 1 trial in end-stage patients with cancers known to express mesothelin, an antigen that is overexpressed in a range of solid tumors.[32] The trial revealed a mesothelin-specific T-cell response in multiple patients, and despite an expected survival of 3–5 mo for all subjects, 6 out of 17 lived for at least 15 mo.

APCure is a new French firm that develops (preclinical stage) live *P. aeruginosa* type III-mediated vectorization of antigens from the Merkel virus, which is responsible for Merkel cell carcinoma.

Perspectives: What is More Exciting than Using Microbes for the Benefit of Mankind?

It is interesting to note that all three of these firms are closely associated with academic laboratories that have explored both the physiology of host/bacteria interactions and bacterial interactions with the immune system and have developed high-level genetic engineering tools. For the Pseudomonas-based vaccine, type III-mediated virulence has been extensively studied by many laboratories throughout the world, but the first demonstration that this bacterium could inject proteins inside immune cells (neutrophils, macrophages, DCs) and that this could help in the development of a novel antigen delivery tool was made by researchers at Grenoble University, from which the APCure firm spun off (Patent number WO2005/049644). This emphasizes the fact that only with in-depth knowledge of the ways in which bacteria interact with their natural hosts and disseminate during infection and a firm understanding of how to engineer bacteria to suit our needs can we develop an innovative therapeutic product. The future of live bacteria-based vaccines is linked to the success of the first line of developed products and to the abilities of scientists to integrate complex data into a synthetic biology approach. Neil S. Forbes have highlighted how synthetic biology techniques can be used to solve many of the key challenges that are associated with bacterial therapies, such as toxicity, stability and efficiency, and to fine-tune their beneficial features, thus enabling the development of "perfect" cancer therapies.[33]

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